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Mononuclear Leukocytes Bind to Specific Hyaluronan Structures on Colon Mucosal Smooth Muscle Cells Treated with Polyinosinic Acid:Polycytidylic Acid

Inter-α-Trypsin Inhibitor Is Crucial to Structure and Function

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Inflammatory bowel disease (IBD) is a chronic disorder whose etiology is linked to triggering events, including viral infections, that lead to immunoregulatory dysfunction in genetically susceptible people. Characteristic pathological changes include increased mononuclear leukocyte influx into the intestinal mucosa as well as mucosal smooth muscle cell (M-SMC) hyperplasia. Virus infection or viral mimic [polyinosinic acid:polycytidylic acid (polyI:C)] treatment of human colon M-SMCs *in vitro* increases cell surface hyaluronan (HA), and nonactivated mononuclear leukocytes bind to virus-induced HA structures by interactions that involve the HA-binding receptor CD44. In this study, confocal microscopy reveals increased HA on poly I:C-treated M-SMC surfaces within 3 hours, arrayed in coat-like structures. By 17 hours, novel, lengthy cable structures are evident, and these are primarily responsible for mediating leukocyte adhesion. Immunohistochemical staining demonstrates components of the inter-α-trypsin inhibitor (IαI) complex in both coat-like and cable structures. M-SMCs co-treated with polyI:C and a polyclonal antibody to IαI display HA in coats but with diminished cables, and they bind significantly fewer leukocytes than M-SMCs treated with polyI:C alone. Western blot data suggest that heavy chains of IαI are specifically associated with cable structures. Staining of tissue sections from patients with IBD demonstrates the presence of HA in inflamed colon tissue, and shows that HA-associated IαI staining increases in the mucosa of inflamed IBD specimens compared to noninflamed sections from the same patient, establishing a probable link between the observations *in vitro* and the progression of the inflammatory process in IBD.
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The origin of inflammatory bowel disease (IBD)[1] is multifactorial. Environmental and microbiological factors initiate and perpetuate an immune response in the intestine of genetically susceptible individuals that results in the clinical manifestations of Crohn's disease and ulcerative colitis. Suggestions that viruses may be one of the environmental stimuli involved in the pathogenesis of IBD have been advanced for some time because of the clinical association of respiratory virus infections with subsequent IBD flares[1] and the specific correlation of the presence of Epstein-Barr virus-infected cells in patient colonic mucosa and IBD.[2]

Pathological changes in IBD include an increase in intestinal mucosal mononuclear leukocytes and hyperplasia of the smooth muscle cells (SMCs) of the muscularis mucosae. The infiltrating leukocytes become activated and contribute to the tissue destruction observed in IBD. Therefore, we have focused on the interaction of mucosal smooth muscle cells (M-SMCs) and leukocytes as a regulatory step in the progression of inflammation.

We previously showed that virus infection dramatically increases the level of mononuclear leukocyte adhesion to M-SMCs through a distinctly different mechanism of interaction from that involved in leukocyte adhesion after treating M-SMCs with inflammatory cytokines.[3] Our data indicate that respiratory syncytial virus, measles virus, and the viral mimic, polyinosinic acid:polycytidylic acid

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(polyI:C), up-regulate leukocyte adhesion primarily through a novel mechanism involving an interaction between hyaluronan (HA) and CD44, a cell surface HA-binding protein, expressed on leukocytes. However, a crucial question that arose from this work is why nonactivated mononuclear leukocytes specifically adhere to HA on virus-induced M-SMCs while they do not adhere to HA constitutively expressed on the same cells.

We have hypothesized that HA-binding proteins synthesized by M-SMCs [CD44, versican, and the protein product of tumor necrosis factor-α-stimulated gene 6 (TSG-6)], as well as the serum proteoglycan inter-α-trypsin inhibitor (IαI) may be involved in the organization of the HA structures that enhance leukocyte adhesion. This study focuses on the possible role of CD44 expressed by the M-SMCs and of IαI in HA-mediated leukocyte adhesion.

CD44, a cell surface glycoprotein expressed on most cells, is known to be a major HA receptor[4] and is expressed by SMCs from a variety of tissue sources.[3,5,6] CD44 has been shown to be important in retention of pericellular matrix chondrocytes[7] and in catabolism of HA by these cells as well as by epidermal keratinocytes.[8] We have previously demonstrated that mononuclear leukocytes bind to polyI:C-induced HA through CD44 on their surfaces. However what role, if any, CD44 on the surfaces of M-SMCs plays in the process of leukocyte adhesion is unknown.

IαI is a member of a family of related proteoglycans of complex structure that are primarily produced by the liver,[9] and serve as serum protease inhibitors. IαI-related proteins are important in the formation of HA-based matrices of certain cells, including those of fibroblasts and mesothelial cells[10] and of the cumulus cells during expansion of the cumulus oophorous in the preovulatory follicle.[11–13] This proteoglycan has a single chondroitin sulfate chain that is covalently bound to a serine residue in the trypsin inhibitor, bikunin.[14] Two other proteins, the heavy chains (members of a gene family, HC1, HC2, and HC3), are covalently linked to the chondroitin sulfate chain of IαI, each through an unusual ester bond between C-terminal aspartate and a 6-hydroxyl on an *N*-acetylgalactosamine residue.[15] When IαI in serum encounters HA within extravascular tissue, as may occur with vascular leakage during an inflammatory response, heavy chains can be covalently transferred from the chondroitin sulfate chains to the HA by a *trans*-esterification reaction and therefore may contribute to the structure of the resulting matrix.

In this study we used confocal microscopy to demonstrate that polyI:C-stimulated M-SMCs elaborate HA in two different forms: patchy coat-like structures adherent to the M-SMC surface, and long cable structures. The cable structures are primarily responsible for binding mononuclear leukocytes via their CD44 receptors.[3] Our data show that CD44 on the M-SMC surface appears to be important for the retention of the coat-like structures whereas an IαI-related protein appears to be a critical molecule for cable formation, and hence leukocyte adhesion.

Materials and Methods

Cell Isolation and Culture

M-SMCs were isolated from human colon specimens obtained within 2 hours after resection from patients undergoing surgery for conditions not involving inflammatory diseases (kindly provided by the Department of Anatomical Pathology, Cleveland Clinic Foundation, Cleveland, OH). The mucosal layer of each colon was removed and cut into strips. The strips were first washed in 50 ml of Hanks' BSS containing 0.15% dithiothreitol (w/v) for 30 minutes, then washed three times in 100 ml of Hanks' BSS containing 1 mmol/L ethylenediaminetetraacetic acid for 1 hour each, and finally washed in Hanks' BSS alone for at least 2 hours with a 100 ml/wash changed every 30 minutes. The tissue samples were then minced, and digested overnight in 100 ml of Hanks' BSS containing collagenase and DNase (0.1 mg/ml each), penicillin (250 U/ml), streptomycin (250 μg/ml), and fungizone (0.625 μg/ml). The liberated cells were filtered from the undigested debris with a tissue screen, cultured in DME/F12 medium supplemented with 10% fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; fungizone, 0.25 μg/ml), and incubated at 37°C in a 5% CO_2 humidified environment. Each 75-cm^2 flask was seeded with the cells obtained from ~125-cm^2 area of original tissue, and contained 15 ml of medium. Two to 3 days after plating, the nonadherent cells were washed away, and the culture fluid replenished. When cell cultures were confluent (~10 days), they were split at a 1:3 ratio. Cultured M-SMCs obtained by this method routinely stain positively for α-SMA actin (antibody from Sigma-Aldrich, St. Louis, MO). M-SMC cultures were used in the first through fourth passages.

U937 cells, originally derived from a human histiocytic lymphoma, were procured from the American Type Culture Collection (Rockville, MD). The cells were grown in suspension culture in RPMI medium containing 5% FBS and routinely subcultured at a 1:5 ratio (~2×10^5 cells/ml) three times per week.

Assay for Leukocyte Adhesion to M-SMCs

Adhesion of U937 cells to M-SMCs was measured as previously described.[3,16] Briefly, M-SMCs were plated into 24-well plates in their appropriate medium (2 to 3×10^4 cells/well in 0.5 ml) 3 to 5 days before the assay, and grown to confluence. Unless otherwise noted in the figure legends, treatment of M-SMCs with polyI:C (20 μg/ml), was done 17 to 24 hours before assay. On the day of the assay, U937 cells (up to 70×10^6 cells/ml) were labeled for 90 minutes at 37°C with 100 μCi of ^{51}Cr as sodium chromate (NEN, Boston, MA) in 1 ml of culture medium. The labeled cells were washed three times with culture medium, counted on a hemacytometer, and resuspended to 10^6 viable (as determined by trypan blue dye exclusion) cells/0.5 ml culture medium. Incubation medium was aspirated from M-SMCs, and 10^6-labeled leukocytes were added to each well. The binding phase of

the assay was done at 4°C for 1 hour. Subsequently, the wells were washed three times with cold medium. The fraction of mononuclear cells bound specifically to HA in each experiment was determined by treating replicate polyI:C-treated cultures to which U937 were bound with testicular hyaluronidase (200 μg/ml final concentration) in medium for 5 minutes at room temperature. All cultures were washed three more times with cold medium. The cells were lysed with 1% Triton X-100, and an aliquot removed for quantitation of radiolabel. The number of U937 cells or monocytes bound per well was calculated from the initial specific activity (cpm/cell). Spontaneous release of chromium from the monocytic cells in control incubations without M-SMCs was typically less than 5%.

Effects of Antibodies to CD44 and IαI on M-SMC Leukocyte Adhesion

PolyI:C-stimulated M-SMC cultures were treated with medium either alone, with affinity-purified polyclonal IαI antibody (1:50 dilution; Novocastra, Newcastle, UK), or with a blocking monoclonal anti-CD44 antibody (from clone A3D8, Sigma) at 20 μl/ml. The antibody-treated M-SMCs and their untreated controls were incubated at 37°C for 18 hours and washed twice before continuing with the leukocyte adhesion assay. The number of leukocytes bound was determined as above.

Fluorescence Histochemistry for Confocal Microscopy

M-SMCs grown on coverslips and treated appropriately for the described experiments, were rinsed with Hanks' BSS, fixed in −20°C methanol for 15 minutes, and air-dried. The coverslips were preincubated with Hanks' balanced salt solution (BSS) containing 2% FBS (30 minutes, 25°C). After removing the medium, the coverslips were incubated with a solution containing biotinylated HA-binding protein (Seikagaku) (5 μg/ml) and an appropriate antibody (monoclonals, 5 to 10 μg/ml; polyclonals, at the recommended dilutions) in Hanks' BSS containing 2% FBS for ~16 hours at 4°C. The coverslips were washed three times with Hanks' BSS, and then incubated with a solution containing fluorescein-tagged streptavidin (1:500) or Texas-red conjugated anti-Ig (H+L) directed to the native species of the primary antibody (1:500) in Hanks' BSS containing 2% FBS. This secondary incubation was done for 1 hour at 25°C. The coverslips were washed three times in Hanks' BSS and mounted to the slides in Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA). The slides were then sealed with nail polish and stored at −20°C.

Confocal images were obtained using a Leica TCS-SP laser-scanning confocal microscope (Leica, Heidelberg, Germany), which is equipped with three lasers and photodetectors that permit detection of three distinct fluorochromes.

IαI, CD44, and Versican Association with the M-SMC Matrix

M-SMCs were plated in 48-well plates and grown to confluence (3 to 4 days). The cells were treated as described in the figure legends, and cell surface-associated IαI, CD44, and versican were determined by a method previously described.[3] Briefly, at the time of the assay, the incubation medium was removed, and the cells were rinsed with DME/F12 containing 2% FBS (wash medium). In some cultures, affinity-purified polyclonal IαI antibody (Novocastra) was added at a dilution of 1:100. In others, monoclonal CD44 antibody (clone A3D8, Sigma) or monoclonal versican antibody (clone 2B1, Seikagaku) was added, each at a concentration of 2 μg/ml in wash medium (100 μl/well). The plates were then incubated at 4°C for 1 hour. After washing the wells three times with cold medium, biotin-conjugated, affinity-purified $F(ab')_2$ fragments from goat anti-rabbit IgG + IgM (H+L) or from goat anti-mouse IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were added to each well at a dilution of 1:1000 in wash medium (100 μl/well), followed by incubation at 4°C for 30 minutes. After washing three times with cold medium, a 1:80 dilution of ^{125}I-streptavidin (Amersham, Arlington Heights, IL) solution was added to each well (100 μl/well), followed by incubation at 4°C for 15 minutes. The wells were then washed four times with cold medium, the cells lysed with 1% Triton X-100, and an aliquot removed for radiolabel quantitation.

Identification of HA-Associated IαI Family Member Proteins by Western Blot Analysis

Confluent cultures of M-SMCs treated with DME/F12 medium containing 5% FBS with or without poly I:C for 18 hours were rinsed three times with Hanks' BSS and treated with *Streptomyces* hyaluronidase (100 mU/ml, 1 ml/75-cm^2 culture; Seikagaku) for 5 minutes at 37°C. The digest supernatant was collected and mixed with an equal volume of Laemmli sample buffer (Bio-Rad, Hercules, CA), and 2-mercaptoethanol (2-ME) was added to a final concentration of 5%. To further characterize the proteins associated with the HA matrix, aliquots were treated with chondroitinase ABC (100 mU/ml) for 1 hour at 37°C or treated with 0.1 mol/L of NaOH at room temperature for 10 minutes before addition of the Laemmli buffer and 2-ME. Equal sample volumes (15 μl) were added to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, followed by electrophoresis. After separation, the samples were electroblotted onto polyvinylidene difluoride-type transfer membranes (Immobilon-P; Millipore, Bedford, MA) at 4°C. Blocking was performed in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) and 5% nonfat dry milk for 2 hours at 25°C. The membrane was incubated with rabbit polyclonal antiserum to IαI (1:1000; DAKO, Indianapolis, IN) in PBST containing 5% nonfat dry milk for 16 hours at 4°C. After five washes with PBST (10 minutes/each), horseradish peroxidase-conjugated secondary antibody

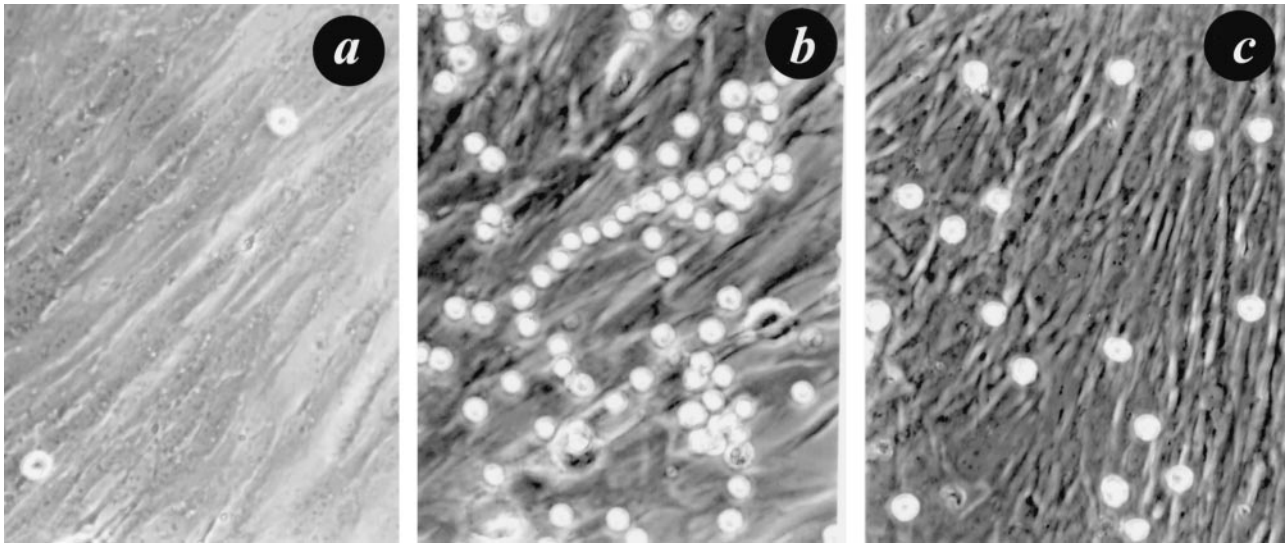


Figure 1. U937 cell binding to polyI:C-induced HA on M-SMCs occurs in clusters. Confluent M-SMCs were treated with DME/F12 medium containing 10% FBS with or without polyI:C for 18 hours at 37°C. U937 cell adhesion was done as described in Materials and Methods and observed by phase contrast microscopy (×100). U937 cells appear as bright spheres on top of the M-SMCs, which are attached to the culture plate. **a**, Medium treated; **b**, polyI:C treated; and **c**, polyI:C treated with hyaluronidase (200 μg/ml) added after adhesion followed by incubation at 25°C for 5 minutes and washing to remove the released U937 cells.

(1:3000 in PBST with 5% milk) was incubated with the membrane for 1 hour at 25°C. Membranes were washed four times with PBST and twice with PBS (10 minutes each). Positive bands were reported by an enhanced chemiluminescence (ECL) detection system (Amersham) according to the kit protocol.

Reagents

All cell culture media, salt solutions, and antibiotics as well as sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels were purchased from Life Technologies, Inc., Grand Island, NY (now Invitrogen Corp). FBS was purchased from Bio-Whittaker. PolyI:C was obtained from Pharmacia, Uppsala, Sweden, or from Sigma. Dithiothreitol and DNase were from Boehringer Mannheim, Indianapolis, IN. Iα1 antibody was purchased from Nova Biomedical, Boston, MA. Biotinylated HA-BP and versican antibody (2B1) were products from Seikagaku America, Ijamsville, MD. Fluorescein isothiocyanate (FITC)-streptavidin and Texas red-IgG were purchased from Jackson ImmunoResearch. West Grove, PA. ^{51}Cr-Sodium chromate was from DuPont-NEN (Boston, MA), and the ^{125}I-streptavidin and the ECL detection kit were from Amersham. Bovine testicular hyaluronidase and the CD44-blocking antibody (A3D8) were from Sigma. Laemmli buffer and the chemiluminescence detection kit were from Bio-Rad.

Results

PolyI:C-Treated M-SMCs Bind Mononuclear Leukocytes via HA

We previously reported that M-SMCs treated with virus or polyI:C (viral mimic) specifically bind mononuclear leukocytes (eg, monocytes, lymphocytes) via HA produced by M-SMCs interacting with CD44 expressed by the leukocytes.[3] Phase contrast micrographs (Figure 1) confirm these findings by demonstrating that in our model system, polyI:C-treated M-SMCs bind U937 monocytic cells abundantly (Figure 1b) compared to unstimulated M-SMCs (Figure 1a), and that treatment with hyaluronidase after adhesion liberates a large portion of the leukocytes (Figure 1c). We have previously demonstrated that the remaining adherent leukocytes are bound to M-SMC surfaces via a VCAM-1-mediated interaction.[3] A finding that can only be appreciated visually, however, is the difference in the pattern of adhesion. The U937 cells that are bound to HA form long chains and three-dimensional clumps (Figure 1b) that are somewhat mobile with gentle movement of the medium, as if tethered to the M-SMC surface. In contrast, leukocytes bound to VCAM-1 are tightly adherent and dispersed on the M-SMC surface (Figure 1c).

PolyI:C Treatment Increases Cell Surface HA on M-SMCs and Is Presented in Two Structures, Pericellular Coats and Cables

Confocal imaging was used to determine how polyI:C-induced HA is arrayed on the M-SMC surface as a function of time after initiation of polyI:C treatment. HA was identified with a biotinylated HA-binding probe (HA-BP), and detected with FITC-streptavidin (green). The cells were also labeled with a monoclonal antibody to CD44, and a Texas-red labeled secondary antibody. Photomicrographs (Figure 2) of fixed M-SMCs that were either untreated (time 0) or treated with polyI:C for different intervals during a 17-hour period (optimal time to induce leukocyte adhesion) show that unstimulated M-SMCs have only a little HA on their surface, and that it is dis-

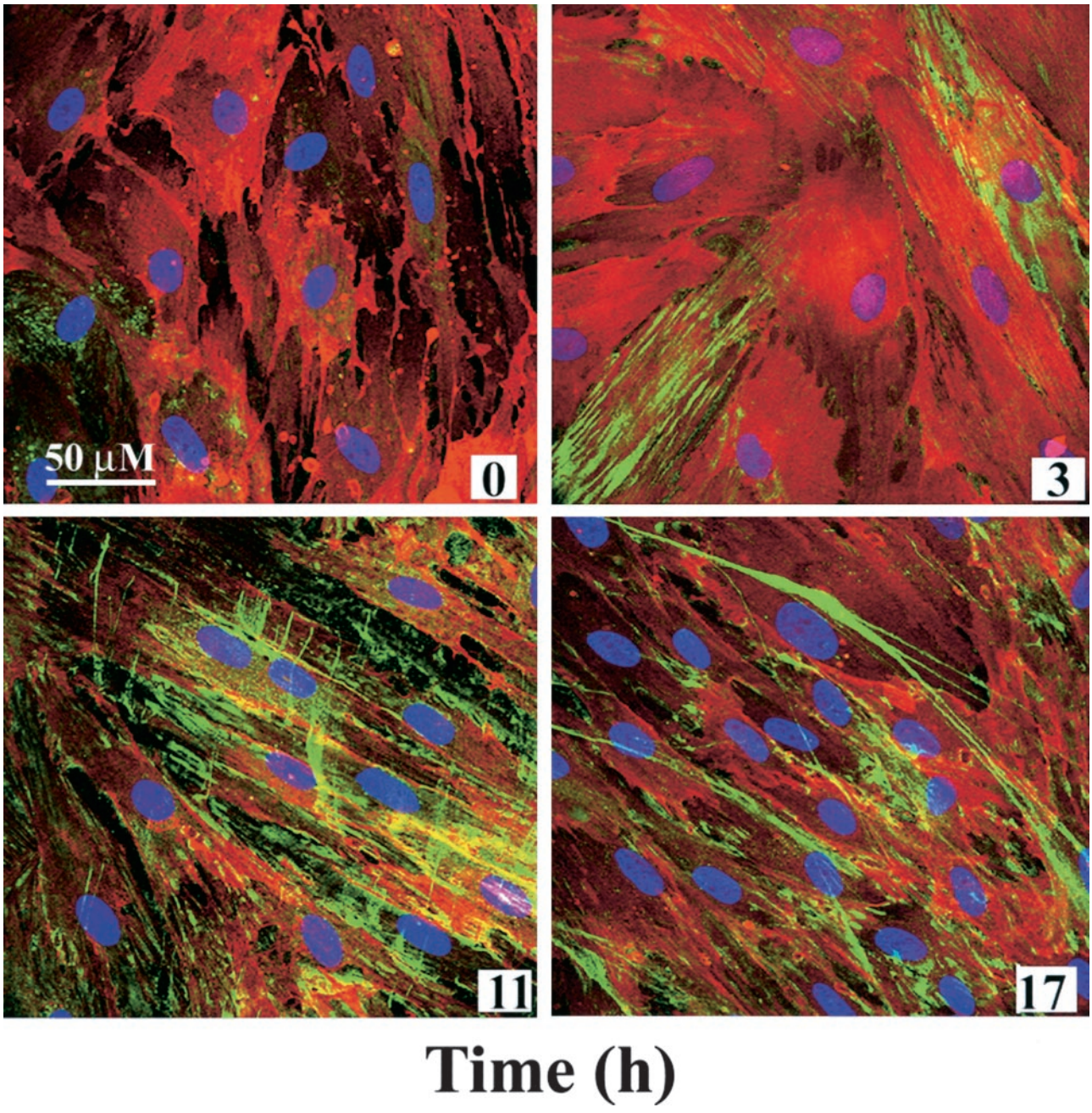


Figure 2. HA is displayed in two distinct structures on polyI:C-treated M-SMCs throughout time. Confluent M-SMCs grown on coverslips were treated with DME/F12 medium containing 10% FBS and polyI:C at 37°C for the times indicated. Coverslips were methanol fixed at intervals throughout 17 hours. Cells were then fluorescently labeled for detection of HA (green, secondary FITC-conjugated reagent), CD44 (red, secondary Texas Red-conjugated antibody), and nuclei (blue, DAPI) (described in Materials and Methods) and observed by confocal microscopy (×20 objective).

played randomly in small patches. In contrast, polyI:C-induced HA forms two obvious structures: pericellular coats and cables of HA that can span several cell lengths. Using the capability of the confocal microscope to detect fluorescence in distinct planes below, at and above the cell surface we consistently observed that induced HA on M-SMC cultures is primarily, but not exclusively, observed at or above the upper cell surfaces. The coat structures appear within 3 hours, and become denser throughout time. By 11 hours, fine strand-like HA structures arising above the cell layer are evident, and by 17 hours thick HA cables are apparent on polyI:C-treated M-SMC cultures. The cables appear to be bundles comprised of thinner HA strands arising from the surfaces of neighboring cells. The M-SMC population is heterogeneous with respect to the expression of HA coats and cables perhaps owing either to differential cell sensitivity to poly I:C, or the presence of different subpopulations of M-SMCs represented in these early (under four passages) cultures derived directly from intestinal mucosa. However, the surface of all M-SMCs stained red, indicating the presence of CD44. Also of note is that CD44 is not

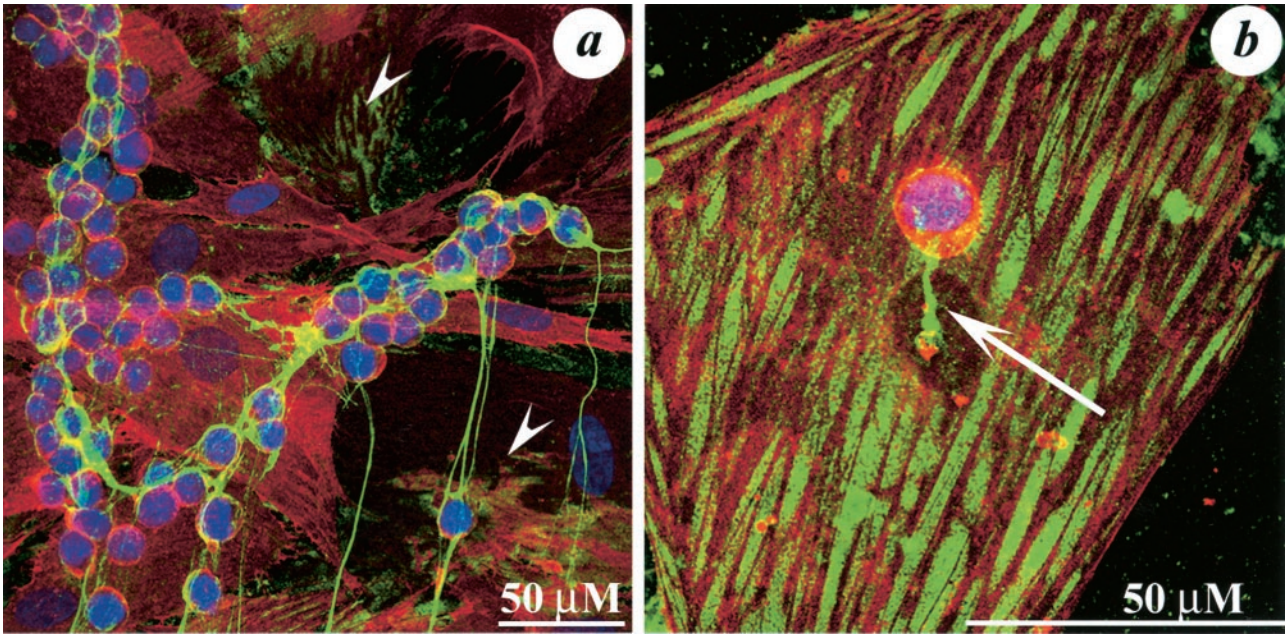


Figure 3. U937 cells bind to polyI:C-induced cable structures but not to coats on the surface of M-SMCs. Confluent M-SMCs grown on coverslips were treated with DME/F12 medium containing 10% FBS and polyI:C for 17 hours at 37°C. The U937 cell adhesion assay was done as described in Materials and Methods. The coverslips were then washed, methanol-fixed, and labeled for detection of HA (green, secondary FITC-conjugated reagent), CD44 (red, secondary Texas Red-conjugated antibody), and nuclei (blue, DAPI) (described in Materials and Methods) and observed by confocal microscopy. **a:** Many CD44-stained U937 cells are attached to the cables, which extend above the adherent monolayer of M-SMCs (×40 objective). **Arrowheads** indicate patches, which are devoid of leukocytes. **b:** A single U937 cell is bound to a cable (**arrow**) rising above the M-SMC nucleus, and not to the patches of HA associated with the M-SMC surface (×100 objective).

identified along the length of the HA cables, while it appears to be in close proximity to the patchy coats.

Leukocytes Preferentially Bind to PolyI:C-Induced HA Cables on M-SMCs

We next asked whether there is a difference between these two evident HA structures in their ability to bind leukocytes. Confocal micrographs (Figure 3) of polyI:C-treated M-SMCs demonstrate that U937 cells selectively bind to HA cables [Figure 3, a and b (arrow)], whereas few to none bind to the pericellular coats [Figure 3, b and a (arrowheads)].

Iαl Is Associated with HA Coats and Cables

Both CD44 and the plasma protein Iαl have been shown to stabilize SMC pericellular HA coats. Versican, another HA-binding protein, is produced by SMCs and is important for their function. Therefore, we measured relative differences in cell surface quantities of these proteins as well as the amounts of HA between unstimulated and polyI:C-treated, live M-SMCs using radiodetection assays. Figure 4A shows a nearly threefold increase in binding of the HA probe and an approximately twofold increase in Iαl antibody binding between the unstimulated and polyI:C-treated M-SMC cultures. This finding was consistent with many different cell isolates. However, in the same assay, versican and CD44 levels, detected by specific antibody binding, were unchanged. In a simultaneous experiment, leukocyte adhesion to M-SMCs increased 10-fold between medium alone and polyI:C-treated conditions (0.12×10^4 and 1.2×10^4 U937 cells bound per culture, respectively).

Because mesenchymal cells do not make Iαl, it is contributed to the M-SMC cultures solely through the fetal bovine serum in the medium. We therefore investigated the importance of this plasma protein to M-SMC HA matrix retention by examining the difference in cable and coat formation in serum-containing *versus* serum-free culture medium. Figure 4B demonstrates that in the presence of medium with serum, poly I:C-treated M-SMCs exhibit greater amounts of both the coat and thickened cable forms of HA as compared to the untreated cultures (Figure 4B, b and a, respectively). However, poly I:C-treatment of M-SMCs in the absence of serum, results in coat formation, with reduced numbers of markedly thinner strands of HA. These strands can be as long as those generated in the serum-containing cultures (Figure 4B, c), but do not coalesce with other HA strands.

PolyI:C-treated M-SMCs, observed by confocal microscopy, displayed Iαl (or a component of the Iαl family) on the M-SMC surfaces (Figure 4B, e). Some of the Iαl was associated not only with HA coats, confirming previous reports, but also stained the cell surface below or in the absence of HA. More significant to our studies however, Iαl co-localized with HA in the cables (Figure 4B, d to f) and can be observed even when distant from the cell surface. Co-localized green and red staining appears yellow in overlaid images.

A

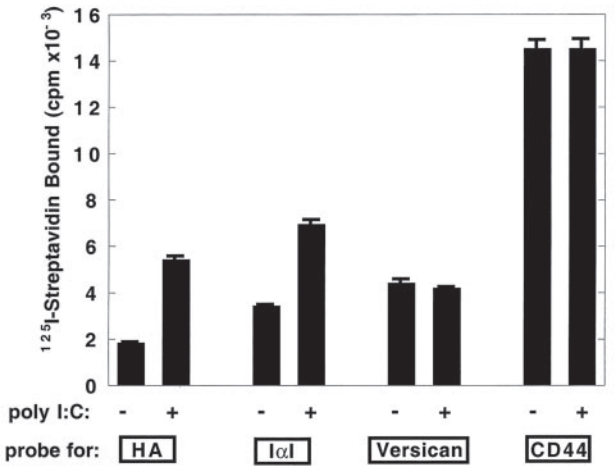
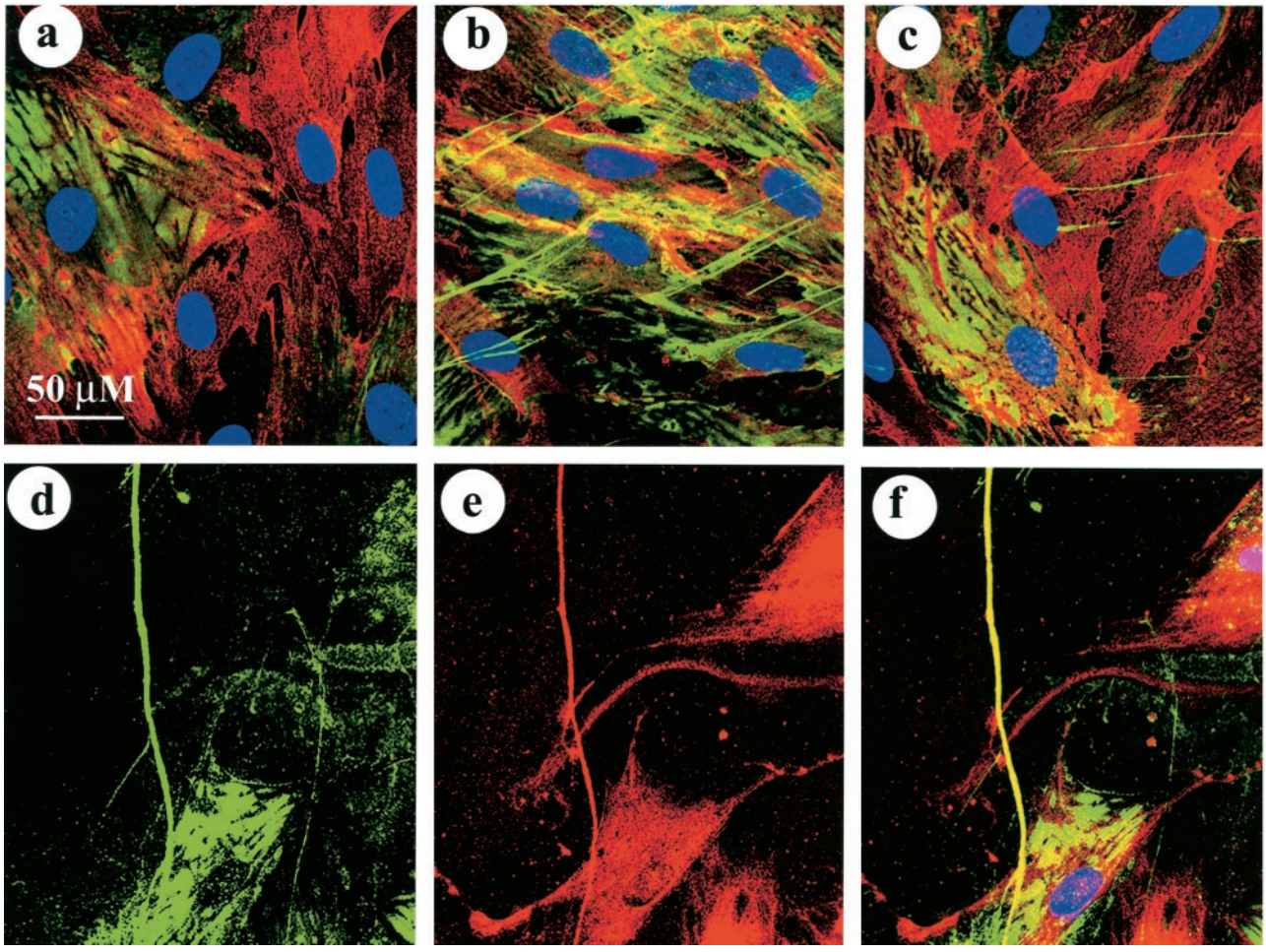


Figure 4. A: Effect of polyI:C on M-SMC expression of cell surface HA, IαI, versican, and CD44. Confluent M-SMCs were treated with DME/F12 medium containing 10% FBS with or without polyI:C for 18 hours at 37°C. Binding of the HA probe, or antibodies to IαI, versican, or CD44 was quantitated as described in Materials and Methods. Values are the mean of triplicate wells ± SEM. **B:** IαI is associated with HA on both the M-SMC surface and the polyI:C-induced cables. Confluent M-SMCs grown on coverslips were treated with DME/F12 medium with (**a**, **b**, **d–f**) or without (**c**) 10% FBS, and with (**b–f**) or without polyI:C (**a**) for 18 hours at 37°C and then methanol-fixed. Cells were then fluorescently labeled for detection of HA (green, secondary FITC-conjugated reagent), IαI (red, secondary Texas Red-conjugated antibody), and nuclei (blue, DAPI) (described in Materials and Methods) and observed by confocal microscopy (×63 objective). Yellow color indicates co-localization of red and green staining.

B



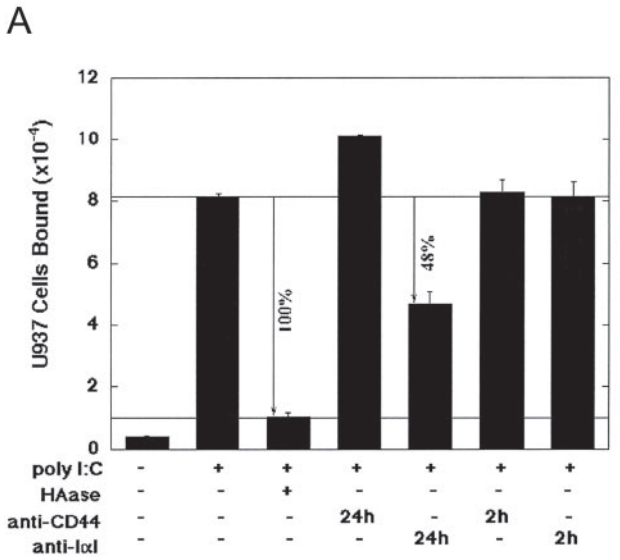
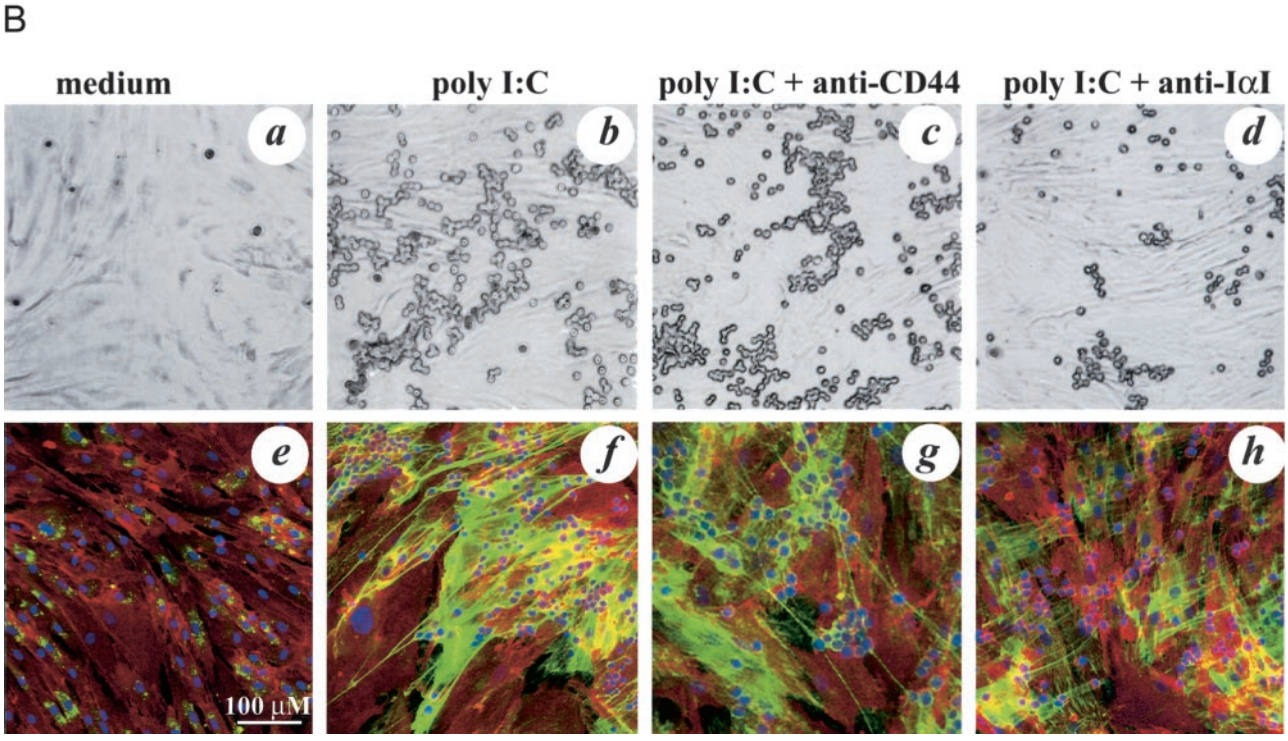


Figure 5. A: Effect of anti-CD44 and anti-IαI antibodies on polyI:C-induced U937 cell adhesion. Confluent M-SMCs were treated with DME/F12 medium containing 10% FBS with or without polyI:C (20 μg/ml). Replicate cultures were co-treated with polyI:C and CD44 antibody (20 μg/ml) or with polyI:C and IαI antibody (1:50). All cultures were incubated for 18 hours at 37°C. In some of the replicate polyI:C-treated cultures, CD44 antibody (10 μg/ml) or IαI antibody (1:50) were added 2 hours before the end of the incubation period. U937 cell adhesion was measured as described in Materials and Methods. A replicate set was also treated with hyaluronidase (200 μg/ml) added after adhesion followed by incubation at 25°C for 5 minutes and washing to remove the released U937 cells. Values are the mean of triplicate wells ± SEM. **B:** Effect of anti-CD44 and anti-IαI antibodies on polyI:C-induced HA cable formation. Confluent M-SMCs were treated with DME/F12 medium containing 10% FBS with or without polyI:C (20 μg/ml). Some replicate cultures were co-treated with polyI:C and CD44 antibody (10 μg/ml) or with polyI:C and IαI antibody (1:50). All cultures were incubated for 18 hours at 37°C. The U937 cell adhesion assay was done as described in Materials and Methods after which the coverslips were washed and methanol-fixed. Light microscopic images were obtained (**a–d**) and then cells were fluorescently labeled for detection of HA (green, secondary FITC-conjugated reagent), CD44 (red, secondary Texas Red-conjugated antibody), and nuclei (blue, DAPI) as described in Materials and Methods and observed by confocal microscopy (**e–h**) (×10 objective).



Antibodies to Iαl Inhibit HA Cable Formation and Leukocyte Adhesion while Antibodies to CD44 Inhibit Pericellular Coat Formation and Enhance Leukocyte Binding

Because Iαl, but not CD44 (Figure 4B), appeared intimately associated with the HA in the leukocyte-binding structures, we asked whether Iαl, or a component of the complex is directly involved in leukocyte adhesion. To do this we investigated the effects of Iαl polyclonal antiserum on the adhesiveness of the HA cables for leukocytes. Replicate cultures of M-SMCs were either untreated, or treated with polyI:C, or co-treated with polyI:C and Iαl antibody, or co-treated with polyI:C and CD44 antibody for 24 hours. Additionally, some polyI:C-treated cultures received an addition of the same quantity of antibody 2 hours before the adhesion assay. The leukocyte adhesion assay (Figure 5A) demonstrated that the Iαl antibody substantially reduced polyI:C-induced leukocyte adhesion (~48% of the hyaluronidase sensitive adhesion) when present during cable formation, whereas CD44 antibody enhanced adhesion (~25%). In multiple experiments, purified nonimmune rabbit IgG, added at the same concentration as the purified Iαl antibody, consistently had no effect on leukocyte adhesion (not shown). Neither affinity-purified antiserum to Iαl nor

monoclonal antibody to CD44 affected U937 cell adhesion when added to cultures after HA cable formation (last 2 hours of incubation).

Similarly, treatment of M-SMCs with poly I:C in the presence of fetal bovine serum that was IαI depleted (produced by incubating serum in the presence of IaI antibody-coated Sepharose beads for 20 hours) resulted in ~25% reduction of HA-mediated leukocyte adhesion as compared to the same treatment in control serum (range, 18 to 30%; three separate experiments) (data not shown).

Light microscopic observation (Figure 5B; a to d) depicts the change in leukocyte adhesion between M-SMCs treated with polyI:C alone, or polyI:C in combination either with CD44 antibody or IαI antibody for the18-hour treatment time. The antibodies were carefully washed out before the adhesion assay to avoid any effect on the U937 cells. We have previously shown, by using this same antibody, that U937 cells and normal mononuclear leukocytes rely on their CD44[3] to bind to poly I:C-induced HA. Cultures that included IαI antibody (Figure 5B, d) have smaller clusters of adhered leukocytes compared to the long chain-like arrays in the polyI:C alone cultures (Figure 5B, b). Confocal microscopic images, Figure 5B, e to h, of the same cultures show the expected generation of HA cables that span many cell widths in response to polyI:C (Figure 5B, f). The inclusion of the CD44 antibody with polyI:C did not decrease cable formation, but did diminish the cell surface patches substantially (Figure 5B, g) demonstrating that the cables are not dependent on coat HA for cell surface attachment. In this case leukocyte binding actually increased, possibly because some HA displaced from the patches becomes incorporated into the cable structures. Inclusion of the IαI antibody resulted in severe truncation of the HA cables Figure 5B, h), the longest only spanning one or two cell widths. In addition the patchy coats were reduced, but not totally displaced.

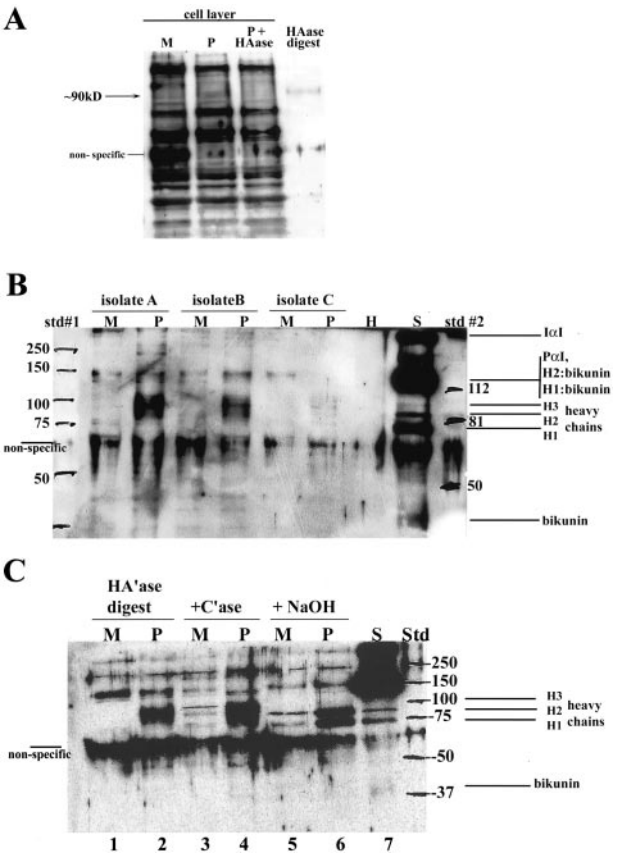


Figure 6. Western blot analysis for HA-associated IαI components in the cell layer of unstimulated and polyI:C-treated M-SMCs. Confluent M-SMCs were treated with DME/F12 medium containing 10% FBS with or without polyI:C (20 μg/ml) for 18 hours. Cells were rinsed and treated with *Streptomyces* hyaluronidase (100 mU/ml) for 5 minutes, processed, and Western blot analysis of IαI done as described in Materials and Methods. **A:** Whole M-SMC extracts from medium-treated (M), poly I:C-treated (P), or poly I:C-treated cells that received a limited hyaluronidase digestion (P + HAase), as well as the cell surface digest (HAase digest) were compared. **B:** Hyaluronidase digests from medium-treated (M) or polyI:C-treated (P) M-SMC cultures from three separate patient isolates, hyaluronidase solution alone (H) or containing fetal bovine serum (S) were compared to two different molecular weight standards (Std #1 and Std #2). **C:** Hyaluronidase (HA'ase) digests from medium-treated (M) or polyI:C-treated (P) M-SMC from a single patient were treated with chondroitinase ABC (C'ase) or NaOH as described in Materials and Methods and processed for Western blot analysis.

IαI Heavy Chains Are Specifically Associated with HA in PolyI:C-Treated M-SMC Cultures

The affinity-purified polyclonal antibody used in these studies recognizes intact IαI family members as well as their component subunits (bikunin, heavy chains 1, 2, and 3). Because radioimmune assays demonstrate increased incorporation of antibody-binding IαI family products on the surfaces of the M-SMCs, we asked whether the increase was specific to any individual species, or generally increased among all of the identified products. Western blot analyses performed on whole cell extracts of medium-treated and polyI:C-treated M-SMCs (Figure 6A) show multiple IαI antibody-reacting bands, including an obvious band of ~90 kd, derived from polyI:C-treated M-SMCs (lane P) that was not apparent in their untreated counterparts (lane M). Minimal digestion of polyI:C-treated M-SMCs with *Streptomyces* hyaluronidase, sufficient for abrogating all HA-based adhesion (100 mU/ml for 5 minutes at 37°C), causes a significant decrease of the ~90-kd band in the cell extract fraction (lane P+ HAase) and a corresponding increase of the protein in the hyaluronidase digest supernatant (lane HAase digest). Figure 6B shows the presence of the ~90-kd band in the hyaluronidase digests from polyI:C-treated M-SMCs from three different patients (P bands), but not in their untreated counterparts (M bands). The intensity of the ~90-kd protein bands differed among the polyI:C-induced M-SMC isolates. As expected, the hyaluronidase solution alone did not display any IαI antibody-reactive bands, and a sample of the fetal bovine serum used for cell growth had a wide variety of bands, including molecular weight species representing IαI; pre-αI; free heavy chains HC1, HC2, and HC3; and bikunin.

The molecular weight of the hyaluronidase-removable band did not correspond to any single component of IαI, yet was not large enough to be the intact molecule. We therefore sought to determine whether the protein band was modified in some way, either by the presence of

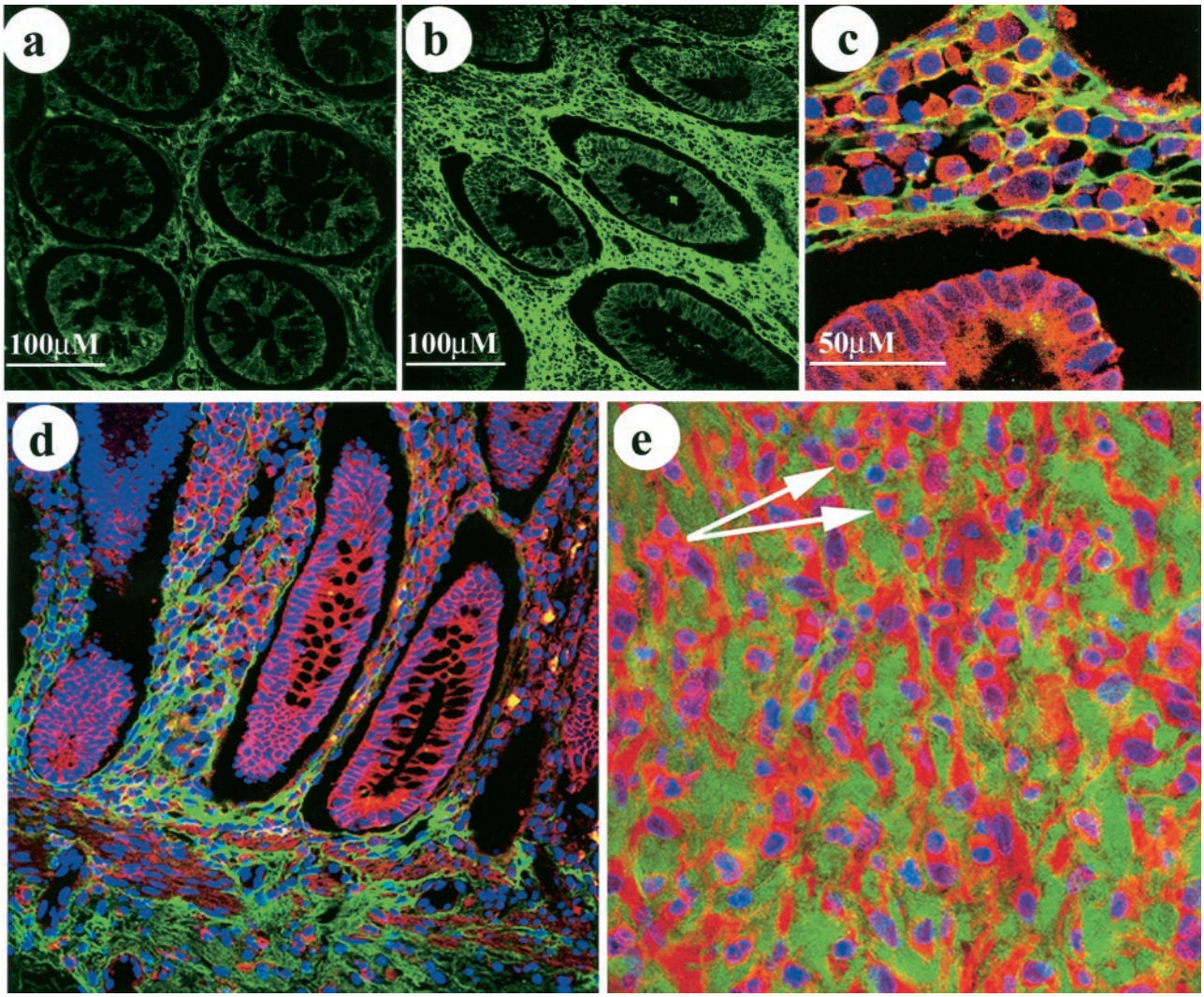


Figure 7. Detection of HA and CD44 in tissue sections from noninflamed and inflamed human colon. Paraffin sections obtained from normal-appearing (**a**) or inflamed (**b**) segments of a resected colon from a patient with ulcerative colitis were fluorescently labeled for detection of HA (green, secondary FITC-conjugated reagent) and nuclei (blue, DAPI) (described in Materials and Methods) and observed by confocal microscopy. Paraffin sections obtained from the same inflamed ulcerative colitis tissue or mildly inflamed (**d**) or inflamed (**e**) segments of a resected colon from a different patient with Crohn's disease were fluorescently labeled for detection of HA (green, secondary FITC-conjugated reagent), CD44 (red, secondary Texas Red-conjugated antibody), and nuclei (blue, DAPI) (described in Materials and Methods) and observed by confocal microscopy [×10 objective (**a**, **b**, **d**); ×40 objective (**c**, **e**)].

chondroitin sulfate moieties with or without bikunin or by aggregation of multiple proteins. Treatment of aliquots from the protein extracts containing the prominent ~90-kd band (Figure 6C, lane 2) with chondroitinase ABC, led to no difference in the apparent molecular weight of that band, yet did reduce the less intense ~150-kd band in the protein extracts, which corresponds in molecular weight to the large pre-αI, HC1:bikunin, and HC2:bikunin bands in the bovine serum (Figure 6C, compare the 150-kd band in lanes 1 and 2 with lanes 3 and 4). Chondroitinase ABC digestion resulted in the appearance of three new faint bands corresponding to the HC1, HC2, and HC3 in the extract of unstimulated cells (Figure 6C, lane 3) and contributed to the intensity of the diffuse ~90-kd band in the extract from the polyI:C-treated cells (Figure 6C, lane 4). Alkali treatment of matched aliquots with NaOH, however, resulted in the disappearance of the ~90-kd diffuse band, and the appearance of two equal and distinct, sharp bands of 74- and 84-kd apparent molecular weights corresponding to the HC1 and HC2 heavy chains of Iαl (Figure 6C, lanes 5 and 6) when compared to the bands of the same molecular weight present in the fetal bovine serum sample (Figure 6C, lane 7).

HA and CD44 Staining Is Increased in Inflamed Colon Tissue from IBD Patients as Compared to Less Affected Areas from the Same Donors

To determine the possible relevance of our *in vitro* observations to the inflammation associated with IBD, we first compared human colon tissue sections stained with the HA-binding probe (FITC) (Figure 7; a to e). Inflamed tissue from patients with ulcerative colitis (Figure 7b) and Crohn's disease (Figure 7e) displayed much more HA staining than the less inflamed colon tissue from the same

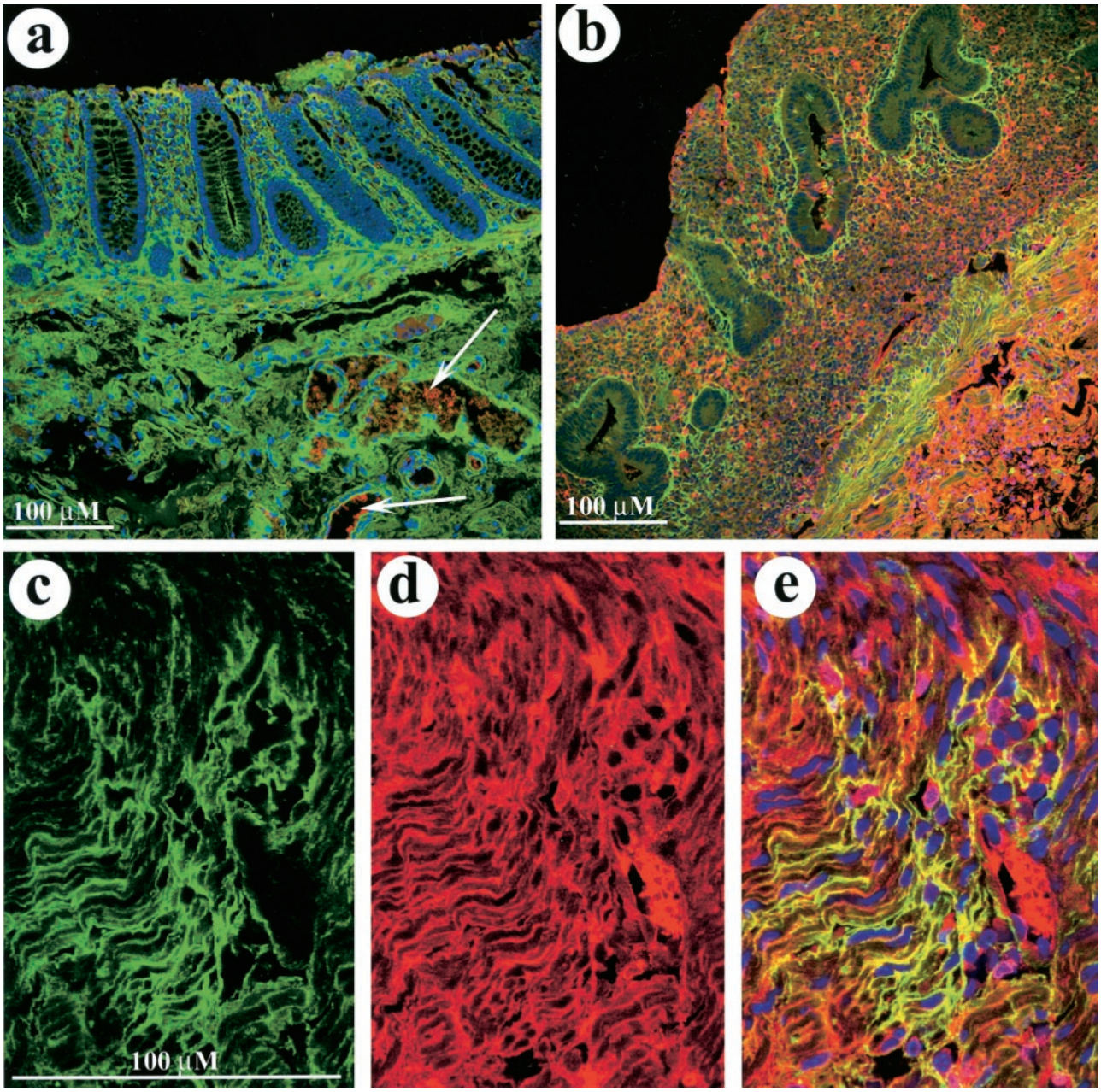


Figure 8. Detection of IαI and HA in tissue sections from noninflamed and inflamed human colon. Paraffin sections obtained from normal appearing (**a**) or inflamed (**b**) segments of a resected colon from a patient with ulcerative colitis were fluorescently labeled for detection of HA (green, secondary FITC-conjugated reagent), IαI (red, secondary Texas Red-conjugated antibody), and nuclei (blue, DAPI) (described in Materials and Methods) and observed by confocal microscopy (×10 objective). Higher magnification (×63 objective–zoom 1) of the muscularis mucosae layer in **b** shows individual detail of green HA staining (**c**), red IαI staining (**d**), and the overlay of the two (**e**) plus blue DAPI nuclear stain to help determine the location of the individual M-SMCs. Yellow color indicates co-localization of red and green staining.

patients (Figure 7, a and d, respectively). HA staining follows a reticular pattern in the loose connective tissue between the crypts of the colon (Figure 7, b and c). CD44 antibody (Texas Red secondary antibody) staining (Figure 7; c, d, and e) confirms the presence of many CD44-positive cells in the leukocyte infiltrate of ulcerative colitis (Figure 7c) and Crohn's disease (Figure 7, d and e) patient tissue. These CD44 cells are in close proximity to HA strands, strikingly observed at higher magnification (Figure 7, c and e; arrows). In early inflammation (Figure 7d) HA appears most prominently associated with the SMCs at the base of the crypts, the muscularis mucosae. Typical of Crohn's disease with advanced inflammation (Figure 7e), the crypt architecture of the colon is lost.

IαI Staining Is Increased in Inflamed Colon Tissue from IBD Patients as Compared to Nonaffected Areas from the Same Donor

Figure 8 depicts sections derived from a surgical specimen removed from a patient with ulcerative colitis that is

labeled with the Iαl antibody (red secondary antibody) and the HA probe (green). Again, a clear qualitative difference was noted between the diseased area and the apparently normal adjacent mucosa from which the sections were made. This was borne out by histological observation of the mucosal layer. In the noninflamed section (Figure 8a) the mucosa is thin and the epithelial cell-lined crypts are continuous and regularly spaced. Leukocyte infiltration is relatively sparse within the lamina propria, and the SMC layer (muscularis mucosae) is only a few cells thick. The submucosa is vascularized loose connective tissue. The only obvious Iαl staining is in the blood vessels of the submucosa (arrows), an expected result because Iαl is a protein contained in serum.

In contrast, the inflamed section (Figure 8b), shows the pathological morphology characteristic of IBD. These features notably include irregularly shaped, distantly spaced crypts, dense leukocyte infiltration, and a greatly thickened muscularis mucosae layer. In this inflamed tissue, and those of other IBD patients, Iαl staining is markedly increased in the mucosal layer. Also, the connective tissue around the blood vessels is more prominently stained as compared to the noninflamed section. Higher power magnification of the hyperplastic muscularis layer reveals a close association between Iαl (Figure 8d) and HA (Figure 8c) staining in the area around the mucosal SMCs (Figure 8e, overlay) individually identified with the help of stained nuclei. Co-localization of HA (green)- and Iαl (red)-stained areas appears yellow.

Discussion

We have described an *in vitro* system in which, on viral insult of colon mesenchymal cells, unstimulated mononuclear leukocytes adhere to HA associated with the muscle cell surface. We previously demonstrated that polyI:C treatment was also able to increase HA production by cultured M-SMCs twofold to threefold.[3] What was not obvious, however, was how such a prevalent structural molecule as HA could become proinflammatory simply because its synthesis was induced by a viral vector. Also puzzling was the observation that unactivated mononuclear leukocytes could bind to this viral-induced HA whereas they would not bind to the constitutive form present on M-SMCs. In addition, other investigators have shown that leukocyte activation is necessary before CD44 binding to HA can occur.[17,18] Yet in our system, the need for activation of CD44 is circumvented.

Confocal microscopic observations provide important evidence that not only the quantity, but also the structure of HA is crucial for its function. PolyI:C-induced HA is displayed on the M-SMCs in patchy coat-like structures as well as in long cable structures, with leukocytes bound mainly to the cables. The HA-binding proteins, CD44 and members of the Iαl family are prominently stained on the surfaces of both the unstimulated and polyI:C-stimulated M-SMCs, and may exist in the absence of HA staining. CD44 is known to be a SMC membrane-expressed receptor. However, the mechanism of association of Iαl family proteins to the M-SMC surface in the absence of HA occurs through an as yet unknown mechanism. Alternatively, this widely used Iαl-directed polyclonal antibody recognizes other proteins on the M-SMC surface. Western blot analysis of hyaluronidase-treated poly I:C-stimulated M-SMC cultures does confirm that most of the Iαl antibody-reacting proteins remain cell associated after removal of HA, at least after the limited digestion used in these experiments.

More pertinent to HA-based leukocyte adhesion, CD44 and Iαl are also closely associated with the HA directly on the M-SMC surfaces. However, of the two groups, only Iαl-related components are identified on the leukocyte-binding cables and only the heavy chains of Iαl are increased in the M-SMC cellular matrix after polyI:C treatment. Here we show, by means of specific antibody interference, the importance of Iαl-related proteoglycans to cable formation and therefore leukocyte adhesion function. We have also observed that M-SMCs stimulated with polyI:C under serum-free conditions, and hence in the absence of Iαl family members, are able to generate thin HA strands, albeit in reduced numbers, but do not form cables, and bind significantly fewer leukocytes.

Western blot analyses demonstrate a striking increase in a particular species of the Iαl complex family with a molecular weight of ~90 kd, which associates with HA on the polyI:C-treated M-SMC surface. Dissociation of the complex by alkali treatment yields only two distinct bands at the molecular weight of the heavy chains (HC1 and HC2) of Iαl. This suggests that the original band was a mix of individual heavy chains complexed either with a small protein (~10 to 20 kd) not recognized by the Iαl antibody or, most likely, with fragments of HA. Because these samples were generated by limited hyaluronidase digestion (5 minutes), a variety of differing molecular weight HA fragments still bound to the heavy chains may be expected, and would account for the diffuse nature of the band observed. These oligosaccharides would be released by the alkali treatment. The specific transfer of heavy chains from Iαl to HA of fibroblasts has been previously shown,[19] and is thought to be a mechanism of matrix stabilization. Our *in vitro* and *in vivo* data support this notion for M-SMCs, but do not explain why heavy chains are incorporated into virus-induced HA, but not the constitutively expressed form.

Iαl-associated proteins are unlikely, however, to be directly involved in leukocyte attachment to HA because addition of Iαl antibody after cable formation has no effect on leukocyte adhesion. The question of how unstimulated mononuclear leukocytes bind through CD44 to the HA cables is still unanswered. One possibility, a physical one, is that the extended HA cables are structures through which many scattered CD44 molecules on the leukocyte surface can be engaged simultaneously, thereby creating interactions between a cell and the matrix that are more stable than those that can be accomplished when fewer CD44 sites are engaged. Alternatively, an accessory-binding molecule incorporated into the HA cables may facilitate CD44 attachment to HA without the leukocyte activation step.

In colon tissue, we consistently observe inflammation-associated increases in HA staining. In addition the HA

forms a reticulum of thick strands that are in close contact with CD44-staining leukocytes around the crypts of inflamed colon sections, confirming that the elements of our *in vitro* model are in place under physiologically relevant conditions.

Changes in IαI-component tissue distribution have been linked to various pathological conditions as diverse as rheumatoid arthritis,[20] kidney stones,[21] malignancies,[22,23] and inflammation.[24] Our *in vivo* histological evidence also demonstrates a striking increase in IαI family proteins associated with a HA meshwork in the mucosal layer of inflamed colon tissue sections that is not observed in noninflamed sections. These observations closely mimic our *in vitro* findings, and add support to our hypothesis that HA-associating proteins confer new, proinflammatory properties on the native molecule.

Our *in vitro* model suggests that HA is synthesized by mucosal SMCs in response to virus, but not in response to cytokines such as tumor necrosis factor-α, interleukin-1, and interleukin-6.[3] HA produced in response to viral stimuli incorporates IαI heavy-chain proteins, which promotes the interaction of mononuclear leukocytes with matrix. As a consequence, interaction of leukocytes with this altered matrix may signal proinflammatory changes, some of which may play a role in the tissue destruction typical of this disease.

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